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Antigenic Epitopes of the Hepatitis A Virus Polyprotein

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Forty-two antigenic domains were identified across the hepatitis A virus (HAV) polyprotein by using a set of 237 overlapping 20-mer synthetic peptides spanning the entire HAV polyprotein and a panel of serum samples from acutely HAV-infected patients. The term "antigenic domain" is used in this study to define a protein region spanned with consecutive overlapping immunoreactive peptides. Nineteen antigenic domains were found within the structural proteins, and 22 were found within the nonstructural proteins, with 1 domain spanning the junction of VP1 and P2A proteins. Five of these domains were considered immunodominant, as judged by both the breadth and the strength of their immunoreactivity. One domain is located within the VP2 protein at position 57–90 aa. A second domain, located at position 767–842 aa, contains the C-terminal part of the VP1 protein and the entire P2A protein. A third domain, located at position 1403–1456 aa, comprises the C-terminal part of the P2C protein and the N-terminal half of the P3A protein. The fourth domain, located at position 1500–1519 aa, includes almost the entire P3B, and the last domain, located at position 1719–1764 aa, contains the C-terminal region of the P3C protein and the N-terminal region of the P3D protein. It is interesting to note that four of the five most immunoreactive domains are derived from small HAV proteins and/or encompass protein cleavage sites separating different HAV proteins. The HAV-specific immunoreactivity of each antigenically reactive peptide was confirmed by using seven HAV seroconversion panels. Collectively, these data demonstrate that HAV structural and nonstructural proteins contain antigenic epitopes that can be efficiently modeled with short synthetic peptides.

INTRODUCTION

Hepatitis A virus (HAV), the causative agent of hepatitis A, is a unique member of the family *Picornaviridae* (Gust *et al.,* 1983; Minor, 1991). The HAV genome is a positive single-stranded RNA, 7.5 kb in length. Similar to all *Picornaviridae,* the HAV genome contains one large open reading frame encoding a polyprotein, which is eventually cleaved into smaller structural and nonstructural proteins (Hollinger and Ticerhurst, 1996).

The antigenic composition of the intact virions has been thoroughly studied. Multiple antigenic epitopes have been detected on the surface of virions and 14S pentamer subunits by using monoclonal antibodies. It was shown that almost all epitopes are located within one immunodominant neutralization site (Lemon and Robertson, 1993; Ping and Lemon, 1992; Stapleton and Lemon, 1987); however, other studies using chimeric picornaviruses have suggested the existence of a secondary neutralization site near the N-terminus of VP1 (Lemon *et al.,* 1992). This finding confirmed a previous observation that peptide derived from the N-terminal regions of VP1 induced HAV neutralizing antibodies (Emini *et al.,* 1985). Most murine HAV monoclonal antibodies compete with one another, and these antibodies can substantially block polyclonal human antibodies from binding HAV in competition immunoassays (Hughes *et al.,* 1984; Stapleton and Lemon, 1987). These observations suggest that there are only a limited number of antigenic epitopes closely placed on the surface of virions. Polyclonal or monoclonal antibodies obtained against native HAV demonstrated only marginal reactivity with denatured capsid proteins (Lemon, 1992). Similarly, antibodies raised to purified capsid proteins VP1, VP2, and VP3 did not efficiently neutralize HAV (Hughes and Stanton, 1985). These observations led to the suggestion that the antigenic epitopes of HAV are mainly conformational discontinuous structures that assemble by higher order interactions between capsid proteins (Lemon and Ping, 1988; Lemon, 1992).

Several approaches have been taken to map the HAV neutralizing epitopes. By cross-linking the Fab fragment of one HAV neutralizing monoclonal antibody to the VP1 protein in intact virions, it was shown that this protein plays an essential role in forming the HAV neutralizing epitope (Hughes *et al.,* 1984). Another approach was to

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identify mutations within the HAV capsid proteins that result in resistance to neutralization with monoclonal antibodies. Neutralization escape mutations for the HAV strain HM175 were identified at Asp_{70}, Gln_{74} of the VP3 protein, Ser_{102}, Val_{171}, Ala_{176}, and Lys_{221} of the VP1 protein (Ping *et al.,* 1988; Ping and Lemon, 1992), and for strain HAS15 at Pro_{65}, Asp_{70}, and Ser_{71} of VP3 and at Asn_{104}, Lys_{105}, and Gln_{232} of the VP1 protein (Nainan *et al.,* 1992).

Synthetic peptides have been extensively used to identify diagnostically relevant antigenic epitopes within the structural proteins (Bosch *et al.,* 1998; Chikin *et al.,* 1991; Kulik *et al.,* 1994, 1995); however, all attempts to find these epitopes have been unsuccessful (Bosch *et al.,* 1998; Chikin *et al.,* 1991; Kulik *et al.,* 1994, 1995). This observation was considered as additional evidence of the strict conformational nature of the HAV capsid antigenic sites. In order to overcome this conformational limitation on modeling HAV epitopes with synthetic peptides a new approach based on selection of desired immunoreactive peptides from random sequence peptide libraries (Houghten *et al.,* 1991; Scott and Smith, 1990) was employed to discover a mimotope that reacted with monoclonal and polyclonal anti-HAV antibodies (Mattioli *et al.,* 1995). A synthetic peptide containing this mimotope very efficiently detected IgM anti-HAV activity in serum specimens of acutely infected patients, although it failed to efficiently detect IgG antibodies (Mattioli *et al.,* 1995).

The HAV nonstructural proteins are also antigenically reactive. An assay based on immune precipitation of proteins translated *in vitro* has been developed to detect antibodies against the HAV nonstructural proteins (Jia *et al.,* 1992). This assay was used to discriminate between antibodies after natural infection and vaccination (Robertson *et al.,* 1992, 1993). Antibodies against P2 proteins were found in all sera from acutely infected patients, whereas chimpanzees vaccinated with inactivated or cell-adapted HAV had no detectable antibodies against P2 products (Robertson *et al.,* 1993). In another study, an enzyme immunoassay (EIA) was developed to detect antibodies to the nonstructural P3C protein. Antibodies to this protein were detected in the serum of primates experimentally infected with virulent HAV and in the serum of acutely infected patients, but antibodies to P3C protein were not detected in the serum from primates following immunization with inactivated HAV (Stewart *et al.,* 1997). These findings demonstrate the utility of HAV nonstructural proteins in differentiating inactivated vaccine-induced immunity from natural infection. However, no further studies on the antigenic composition of these proteins were conducted.

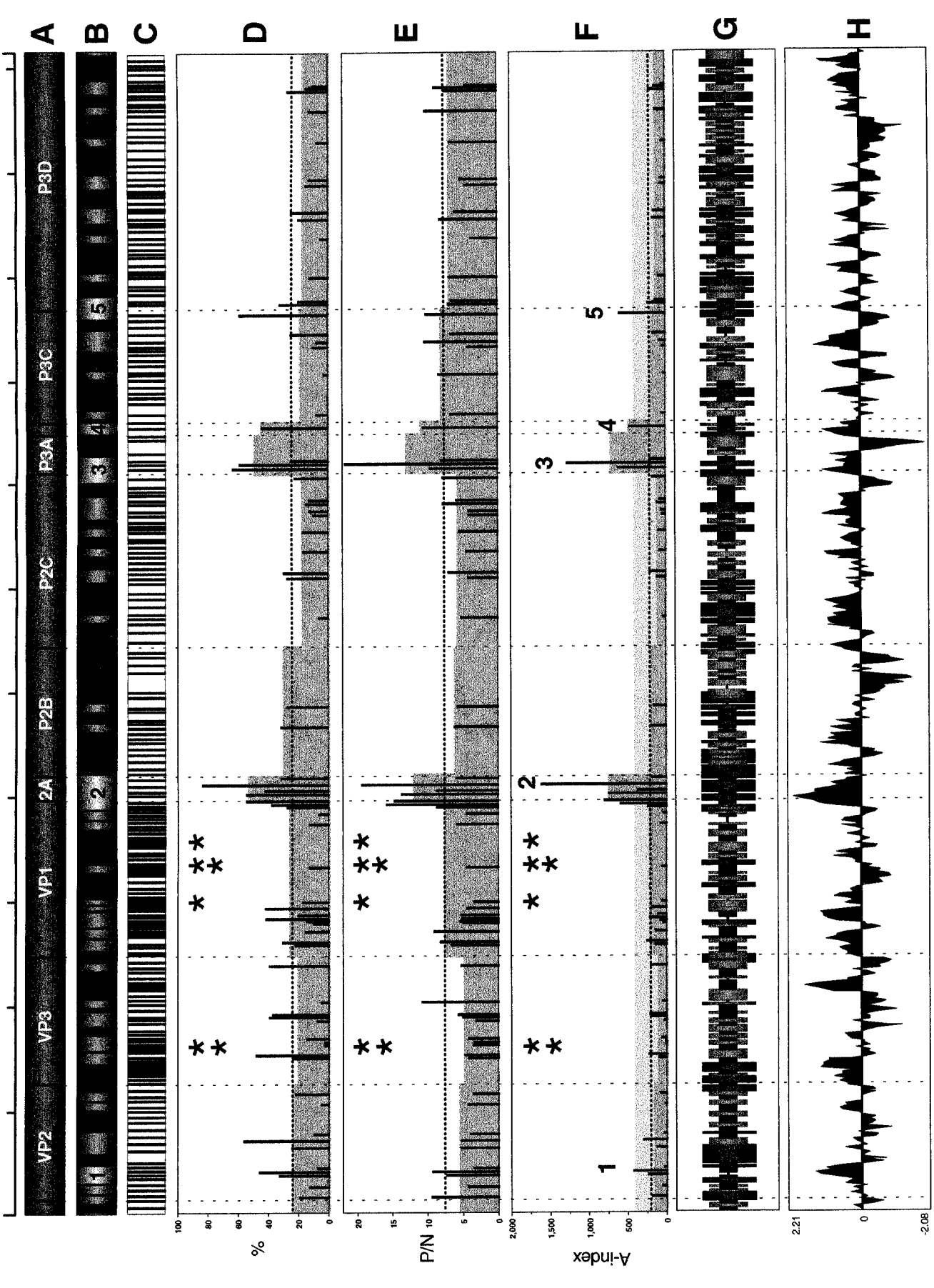
HAV infection is diagnosed by the detection of IgM or IgG antibodies to the capsid proteins (Bradley *et al.,* 1977). Because of apparently poor antigenic reactivity, HAV recombinant proteins and synthetic peptides have not been successfully employed in an EIA for the detection of anti-HAV activity. Currently, the only available source of immunoreactive proteins for assay development is inactivated HAV derived from cell culture, which is currently used by all commercial companies that manufacture anti-HAV tests. In addition to the inconvenience and cost associated with the production, purification, and standardization of cell culture-derived HAV antigen, current commercially available assays are unable to discriminate between natural infections and vaccine induced immunity, as emphasized in several publications (Jia *et al.,* 1992; Robertson *et al.,* 1992, 1993; Steward *et al.,* 1997), since these tests utilize intact HAV and therefore will detect antibodies in either case.

The present study provides, for the first time, a comprehensive analysis of the antigenic composition of the entire HAV polyprotein by using a large number of synthetic peptides. The results demonstrate the existence of several broadly and strongly antigenically reactive regions, many of which have significant diagnostic potential within both structural and nonstructural proteins.

RESULTS AND DISCUSSION

Peptide design

The sequence of the polyprotein of the HAV from strain HM175 (Cohen *et al.,* 1987) was used to design all synthetic peptides in this study. A total of 237 overlapping 20-mer peptides were synthesized. Almost the entire sequence of the HAV HM175 polyprotein was spanned with these peptides with only a few exceptions: four small hydrophobic regions at positions 106–109, 1011–1029, 1471–1476, and 1546–1547 aa were not spanned with peptides. In addition, in three cases peptides were derived from adjacent nonoverlapping hydrophobic regions in such a way that positions 980–981, 1087–1088, and 1107–1108 aa were represented only at the termini of peptides. These seven regions are shown in Fig. 1C as larger than average gaps between small vertical bars. Some regions where antigenic activity has been shown previously (Emini *et al.,* 1985; Kulik *et al.,* 1994, 1995; Nainan *et al.,* 1992; Ping *et al.,* 1988; Ping and Lemon, 1992) or could be predicted with high probability (Jameson and Wolf, 1988) based on protein chain flexibility (Karplus and Schultz, 1985), hydrophylicity (Kyte and Doolittle, 1982), and/or secondary structure (Chou, 1990) were spanned with a higher peptide density. These regions can be identified in Fig. 1C as clusters of small vertical bars. For example, two clusters of peptides within the N-terminal part of the VP3 protein (Fig. 1C) were generated around an antigenic epitope that had been previously reported (Kulik *et al.,* 1995) and around residues Asp_{315} and Gln_{319} (shown by two asterisks in Fig. 1C) that have been found to be essential to the HAV major conformational neutralizing epitope (Ping *et al.,* 1988; Ping and Lemon, 1992). Within the VP1 protein, peptide clusters were synthesized around an antigenic


A
B
C
D
E
F
G
H
VP2
VP3
VP1
2A
P2B
P2C
P3A
P3C
P3D
200
400
600
800
1000
1200
1400
1600
1800
2000
%
P/N
A-index
2.21
0
-2.08

epitope that had been previously found to elicit neutralizing antibody (Emini *et al.,* 1985; Lemon *et al.,* 1992); around amino acid residues Ser_{593}, Val_{662}, Ala_{667}, and Lys_{712} (indicated by asterisks in Fig. 1C), which were shown to be important for the functional activity of the major HAV neutralizing antigenic epitope (Ping and Lemon, 1992); and around the region at position 714–752 aa that was shown to be exposed on the surface of HAV particles (Robertson *et al.,* 1989). Surface exposure is generally considered to be an important indicator of potential antigenic reactivity (Emini *et al.,* 1985).

On average, every amino acid of the HAV HM175 polyprotein was represented 2.1 times in these synthesized peptides. Peptides were overlapped on average by 10 aa. In peptide clusters, peptides were often overlapped by 14–18 aa. Among 237 peptides, 110 were derived from structural proteins and 127 were derived from nonstructural proteins. Thus, taking into consideration the difference in the size of the HAV structural region (791 aa) and the nonstructural region (1436 aa), the density of peptides derived from structural proteins is approximately 1.6 times greater than that from nonstructural proteins.

Immunoreactivity of peptides derived from structural proteins

The antigenic reactivity of the HAV structural proteins has been extensively investigated using synthetic peptides (Bosch *et al.,* 1998; Chikin *et al.,* 1991; Kulik *et al.,* 1994, 1995), and strong antigenic regions modeled with short synthetic peptides or with recombinant proteins were not ascertained. In this study, however, a number of strong antigenic epitopes were discovered. These contradicting results may be explained by: (1) the use of acute-phase serum specimens in the present study, whereas all previous studies employed convalescent-phase serum specimens or polyclonal or monoclonal antibodies obtained against HAV particles and (2) a thorough scan of each protein by using significantly overlapping peptides.

VP4–VP2 proteins. Among 21 peptides derived from the VP4–VP2 proteins, 10 were found immunoreactive with acute anti-HAV serum specimens, as indicated by the vertical bars in Fig. 1. These immunoreactive peptides were used to identify 6 antigenic domains, as indicated by alternating consecutive rows of the same shade in Table 1. In this study, an antigenic domain is defined as a protein region spanned with immunoreactive consecutive overlapping synthetic peptides or a single peptide separated from other antigenic regions by nonimmunoreactive peptides. For example, four domains of the VP4–VP2 protein, at positions 15–34, 35–54, 194–213, and 214–233 aa, were identified with only one peptide each. Two domains at positions 57–90 and 110–156 aa were spanned with three peptides each (Table 1). Three peptides (1210, 1211, and 1216) from the two largest domains within the VP4–VP2 region demonstrated broad immunoreactivity with acute anti-HAV-positive serum specimens. These peptides immunoreacted with more than 30.0% of tested serum specimens with a range from 32.6 to 56.1%. All three peptides contain antigenic epitopes that have not been previously reported. Overlapping peptides, 1215 and 1216, demonstrated approximately a two times higher background immunoreactivity with anti-HAV-negative serum specimens (data not shown). Also, peptide 1216 demonstrated nonspecific immunoreactivity with 4 of 46 anti-HAV negative specimens (Table 1). Thus, the region at position 110–143 aa, as modeled with two consecutive overlapping synthetic peptides, exhibited HAV nonspecific reactivity under the experimental conditions that were uniformly applied to all peptides (see Materials and Methods).

Several peptides comprising regions 65–85 aa (Chikin *et al.,* 1991), 92–122 aa (Kulik *et al.,* 1994, 1995), 103–122 aa (Kulik *et al.,* 1994), and 119–130 aa (Bosch *et al.,* 1998) from the HAV VP2 protein and region 1–23 aa from the HAV VP4 protein have been synthesized and tested with anti-HAV-positive serum specimens previously. Among these peptides, only one, containing sequence 65–85 aa, was found to be not immunoreactive (Chikin *et al.,* 1991). However, this peptide significantly overlaps with the strongly immunoreactive peptide 1211, which contains this sequence (Table 1). The overlapping peptides 92–122 (Kulik *et al.,* 1994, 1995)and 103–122 aa (Kulik *et al.,* 1994) were found immunoreactive with convalescent-phase serum specimens and monoclonal antibodies obtained against the whole virus. These two peptides share sequences with peptide 1214, which is not immunoreac-

FIG. 1. Antigenic reactivity of synthetic peptides. (A) HAV polyprotein; (B) antigenic domains; 1–5, most immunoreactive antigenic domains; (C) vertical bars show the location of the center of each synthesized peptide; (D) percentage of serum specimens that are immunoreactive with each peptide; shaded area shows the average percentage immunoreactivity for peptides derived from each protein and dotted line shows the average percentage immunoreactivity for all peptides; (E) average *P*/*N* ratio calculated for all serum specimens immunoreactive with each synthetic peptide, where *P* represents the OD_{493} of anti-HAV-positive specimens and *N* represents the OD_{493} of negative specimens; shaded area shows the average *P*/*N* value for peptides derived from each protein and the dotted line shows the average *P*/*N* for all peptides; (F) the A index for each peptide (see Materials and Methods); dark shaded area shows the average A index for peptides derived from each protein, and the light shaded area shows the value that is two times greater than the average A index for the entire polyprotein shown by dotted line; (G) predicted secondary structure (Chou, 1990): black bars, alpha-helix; shaded bars, beta-structure; small black bars, beta-turns; horizontal line, random coil; (H) hydropathic plot (Kyte and Doolittle, 1982): hydrophilic regions are shown above the line.

TABLE 1

Antigenic Reactivity of Synthetic Peptides Derived from the HAV Structural Proteins

Peptide	Sequence	Position aa	Acute[1] sera	P/N[2] acute sera	NHS[3]
VP4-VP2					
YK-1206	GLDHILSLADIEEEQMIQSV	15-34	8/42 19.1%	9.46 (3.01-27.07)	0/46
YK-1208	DRTAVTGASYFTSVDQSSVH	35-54	5/41 12.2%	4.29 (3.27-5.54)	0/46
YK-1210	EVGSHQVEPLRTSVDKPGSK	57-76	15/46 32.6%	7.31 (3.93-20.47)	0/48
YK-1211	EPLRTSVDKPGSKKTQGEKF	64-83	21/46 45.7%	9.33 (3.37-27.92)	0/48
YK-1212	DKPGSKKTQGEKFFLIHSAD	71-90	3/41 7.3%	3.5 (3.04-3.96)	0/46
YK-1215[4]	LYNEQFAVQGLLRYHTYARF	110-129	10/41 24.4%	5.29 (3.41-8.83)	0/46
YK-1216[4]	HTYARFGIEIQVQINPTPFQ	124-143	23/41 56.1%	5.3 (3.42-10.79)	4/46
YK-1217	INPTPFQQGGLICAMVPGDQ	137-156	4/41 9.8%	4.03 (3.94-4.12)	0/46
YK-1222	HFKDPQYPVWELTIRVWSEL	194-213	1/41 4.9%	4.29	0/46
YK-1224	NIGTGTSAYTSLNVLARFTD	214-233	9/41 22.0%	3.53 (3.09-4.17)	0/46
VP3					
YK-1235	SDPSQGGGIKITHFTTWTSI	283-302	11/46 23.9%	4.2 (3.0-4.95)	0/48
YK-1236	GGIKITHFTTWTSIPTLAAQ	289-308	22/46 47.8%	4.68 (3.1-9.33)	0/48
YK-1241	QFPFNASDSVGQQIKVIPVD	308-327	1/41 2.4%	3.56	0/46
YK-1242	FNASDSVGQQIKVIPVDPYF	311-330	1/41 2.4%	3.1	0/46
YK-1243	SDSVGQQIKVIPVDPYFFQM	314-333	1/41 2.4%	3.47	0/46
YK-1244	IKVIPVDPYFFQMTNTNPDQ	321-340	6/41 14.6%	4.2 (3.55-5.2)	0/46
YK-1248	FWRGDLVFDFQVFPTKYHSG	355-374	2/41 7.3%	3.16; 3.2	0/46
YK-1249[4]	FDFQVFPTKYHSGRLLFCFV	362-381	18/46 39.1%	5.03 (3.07-7.03)	0/48
VP3					
YK-1250[4]	FPTKYHSGRLLFCFVPGNEL	367-386	17/46 37.0%	5.68 (3.4-7.92)	0/48
YK-1252	GITLKQATTAPCAVMDITGV	391-410	2/41 4.9%	3.67; 17.95	0/46
YK-1261[4]	VASHVRVNVYLSAINLECFA	461-480	16/41 39.0%	5.27 (3.72-7.9)	1/46
VP1					
YK-1265	TTVSTEQNVPDPQVGITTMK	501-520	9/41 22.0%	6.7 (3.23-9.57)	0/46
YK-1266	QNVPDPQVGITTMKDLKGKA	507-526	14/46 30.4%	8.25 (3.27-18.30)	0/48
YK-1268	NRGKMDVSGVQAPVGAITTI	527-546	7/46 15.2%	9.1 (6.07-15.25)	2/48
YK-1271	ITTIEDPVLAKKVPETFPEL	543-562	4/41 9.8%	4.97 (3.33-7.76)	1/46
YK-1272	EDPVLAKKVPETFPELKPGE	547-566	6/41 14.6%	5.4 (4.87-6.12)	1/46
YK-1273[4]	AKKVPETFPELKPGESRHTS	552-571	19/46 41.3%	5.15 (3.15-8.58)	3/48
YK-1274	FPELKPGESRHTSDHMSIYK	559-578	9/46 19.6%	5.31 (3.0-8.47)	1/48
YK-1276[4]	DHMSIYKFMGRSHFLCTFTF	572-591	17/41 41.5%	4.38 (3.15-5.62)	0/46
YK-1279	HFLCTFTFNSNNKEYTFPIT	584-603	7/41 17.1%	3.43 (3.11-3.67)	1/46
YK-1290	TPVGLAVDTPWVEKESALSI	651-670	6/48 12.5%	4.48 (3.0-6.44)	1/51
YK-1307	LSFSCYLSVTEQSEFYFPRA	734-753	6/48 12.5%	5.88 (4.17-7.64)	0/51
YK-1310	PLNSNAMLSTESMMSRIAAG	754-773	2/48 6.3%	3.68; 5.23	0/51
YK-1312	MSRIAAGDLESSVDDPRSEE	767-786	13/48 27.1%	8.7 (4.44-25.9)	2/51
YK-1313	AGDLESSVDDPRSEEDKRFE	772-791	18/48 37.5%	15.79 (3.0-108.06)	3/51
YK-1314	VDDPRSEEDKRFESHIECRK	779-798	26/48 54.2%	14.68 (3.53-89.96)	1/51

Note. Alternating boldface and italic type represent individual antigenic domains.

[1] The number of positive serum samples divided by the total number of tested sera and percentage of immunoreactive sera.

[2] Mean *P/N* value; number in parentheses indicates the range of *P/N* values.

[3] Normal human sera.

[4] Background reactivity with anti-HAV-negative sera is two times greater than that for other peptides.

tive and includes the sequence 87–106 aa, and peptide 1215, which is immunoreactive and comprises the sequence 110–129 aa. It is conceivable that the overlapping region between these three immunoreactive peptides at position 110–122 aa may represent at least an essential part of a common antigenic epitope. The other immunoreactive peptide, described by Bosch *et al.* (1998) and comprising region 119–130 aa, essentially overlaps immunoreactive peptide 1215 containing the sequence 110–129 aa (Table 1). Thus, the common antigenic epitope modeled with these peptides may be located within the region at position 119–129 aa. It is interesting to note that the last region contains residue Tyr_{123}, which was found exposed on the surface of HAV particles (Robertson *et al.,* 1989). Peptide comprising the entire HAV VP4 protein (1–23 aa) has been synthesized and shown not to be immunoreactive with anti-HAV-positive convalescent-phase serum specimens (Chikin *et al.,* 1991). Two peptides that overlap this sequence were used in this study. One peptide comprising the sequence 1–19 aa was not immunoreactive, while the other peptide, 1206, was immunoreactive (Table 1). Since the peptide containing the region 1–23 aa was tested by using convalescent-phase serum specimens, which are, in general, less immunoreactive than the acute specimens used in the present study, it is impossible to determine

whether the VP4 region at position 15–23 aa, as represented within peptide 1206 (Table 1), contains an antigenic epitope or the antigenic reactivity is attributed entirely to the N-terminal part (24–34 aa) of the VP2 protein included within this peptide.

VP3 protein. Among 37 peptides spanning the entire HAV VP3 protein, 11 peptides were found to be immunoreactive (Fig. 1). An analysis of overlapping immunoreactive peptides allowed the identification of five antigenic domains within this protein (Table 1). Two C-terminal domains of the VP3 protein were identified with one peptide each, while the N-terminal domain was identified with two overlapping peptides. Two central domains were identified with three and four immunoreactive synthetic peptides (Table 1), respectively. Four peptides (1236, 1249, 1250, and 1261) that belong to three different antigenic domains were found to be broadly immunoreactive. These peptides demonstrated immunoreactivity with more than 30.0% of anti-HAV-positive serum specimens. Therefore, three domains at positions 283–308, 355–386, and 461–480 aa also contain the most immunoreactive epitopes, as modeled with synthetic peptides. Three peptides derived from regions at positions 362–386 and 461–480 aa demonstrated high background immunoreactivity (Table 1). Peptide 1261 demonstrated nonspecific immunoreactivity with 1 of 46 anti-HAV-negative specimens under the experimental conditions that were uniformly applied to all synthetic peptides (see Materials and Methods).

Peptides derived from regions at positions 290–302 aa (Kulik *et al.,* 1994), 321–330 aa (Chikin *et al.,* 1991), 355–366 aa (Bosch *et al.,* 1998), and 382–395 aa (Kulik *et al.,* 1994, 1995) were previously tested for antigenic reactivity with human anti-HAV-positive convalescent-phase serum specimens and with monoclonal antibodies. Except for peptide containing the sequence 321–330 aa, three of these peptides were found to be immunoreactive. It is interesting that the sequence of the nonimmunoreactive peptide was represented within two immunoreactive overlapping peptides, 1243 (314–333 aa) and 1244 (321–340 aa), described in the present study (Table 1). Conversely, the peptide containing the sequence 382–395 aa has been previously reported as immunoreactive (Kulik *et al.,* 1994, 1995); however, peptide 1251 (380–399 aa), which contains this complete sequence, was not found to be immunoreactive in our study (Table 1). Peptide 1250 (367–386 aa) and 1252 (391–410 aa) are partially overlapped with an antigenic epitope at position 382–395 aa previously identified by Kulik *et al.* (1995). However, these peptides do not appear to model this epitope because neither peptide contains the complete sequence of this epitope. The antigenic reactivity of two other peptides containing sequences at positions 290–302 aa (Kulik *et al.,* 1994) and 355–366 aa (Bosch *et al.,* 1998) was confirmed in the present study with peptides 1235 (283–302 aa) and 1248 (355–374 aa) (Table 1).

Three weakly immunoreactive peptides (1241, 1242, and 1243) contain residues Asp_{315} and Gln_{319}, which have been found to be essential residues of the HAV major conformational neutralizing epitope (Lemon, 1992; Ping *et al.,* 1988; Ping and Lemon, 1992). These peptides also contain the region at position 308–321 aa, known to elicit antibody that recognizes HAV particles (Lemon, 1992).

VP1 protein. Among 52 synthetic peptides spanning the entire HAV VP1 protein, 15 were found to be anti-HAV immunoreactive (Fig. 1). These peptides identify nine antigenic domains (Table 1). Six domains at positions 527–546, 572–591, 584–603, 651–670, 734–753, and 754–773 aa were identified with only one peptide each. The N-terminal domain at position 501–526 aa was identified with 2 peptides, while the C-terminal domain at position 767–798 aa was identified with 3 peptides. One domain at position 543–578 aa was identified with 4 peptides (Table 1). Five peptides (1266, 1273, 1276, 1313, and 1314) derived from four antigenic domains were found to be immunoreactive with more than 30.0% of anti-HAV-positive serum specimens. However, 2 of these broadly immunoreactive peptides demonstrated a two times higher background reactivity with anti-HAV-negative specimens compared to all the other peptides (Table 1). Moreover, 1 of these 2 peptides, 1273, was shown to immunoreact with 3 of 48 anti-HAV-negative specimens, thus demonstrating nonspecific reactivity. Another broadly immunoreactive peptide, 1313, also demonstrated nonspecific reactivity with about 6.0% of anti-HAV-negative serum specimens (Table 1). It should be noted that 10 of 15 VP1-derived immunoreactive peptides were shown to exhibit HAV nonspecific antigenic reactivity compared to only 2 peptides among all peptides derived from both VP2 and VP3 proteins (Table 1). It should be emphasized, however, that the assay experimental conditions were uniformly applied to all synthetic peptides without regard to optimizing assay conditions for each peptide (see Materials and Methods). It is likely that with proper optimization, especially for the concentration of each peptide adsorbed to microtiter wells, the nonspecific immunoreactivity might be significantly reduced or eliminated.

A number of peptides derived from the HAV VP1 protein were previously tested for antigenic reactivity. Peptide containing the sequence at position 502–516 aa was shown to elicit HAV neutralizing antibodies (Bosch *et al.,* 1998; Emini *et al.,* 1985; Lemon *et al.,* 1992). This peptide was also recently found to be immunoreactive with anti-HAV antibodies (Bosch *et al.,* 1998). This finding contradicts a previous study in which no antigenic reactivity was found associated with a peptide of the same sequence (Kulik *et al.,* 1994, 1995). In our experiments, the peptide 1265, completely comprising the sequence of this peptide, was shown to immunoreact with 22.0% of acute-phase anti-HAV-positive serum specimens (Table 1), thereby confirming the presence of an antigenically

reactive epitope within this region of the HAV VP1 protein.

Another antigenic region within the HAV VP1 protein was previously found by using a set of three peptides containing the sequence 606–630 aa (Kulik *et al.,* 1994, 1995). However, this observation was not confirmed by results obtained with peptide 609–631 aa (Chikin *et al.,* 1991), which was found not to be immunoreactive. In our study, we also could not confirm the presence of an antigenic epitope(s) within this region, since peptide 1284 containing the sequence 606–625 aa did not show any HAV-specific antigenic reactivity.

Additionally, a set of peptides derived from regions at position 492–508, 564–577, 589–599, 599–614, and 767–789 aa was previously tested with anti-HAV-positive serum specimens (Chikin *et al.,* 1991). None of these peptides, however, was found to be immunoreactive. In this study, two peptides, 1264 (490–509 aa) and 1283 (598–617 aa), comprising two of these regions, were also nonimmunoreactive, thus confirming the absence of antigenic reactivity within regions 492–508 and 599–614 aa. However, peptide 1274 (559–578 aa), 1279 (584–603 aa), 1312 (767–786 aa), and 1313 (772–791 aa), which completely comprised sequences from the other three regions, were found to be immunoreactive, with peptide 1313 demonstrating the strongest and broadest immunoreactivity (Table 1). Interestingly, immunoreactive peptide 1279 contains Ser_{102}, which was shown to be an important residue of the major HAV neutralizing antigenic epitope (Lemon, 1992; Ping *et al.,* 1988; Ping and Lemon, 1992). Immunoreactive peptide 1290 (Table 1) contains another two amino acids, Val_{171} and Ala_{176}, also shown to be essential for the activity of this neutralizing epitope (Lemon, 1992; Ping *et al.,* 1988; Ping and Lemon, 1992).

Peptides 1304, 1305, 1306, and 1307 are located within the region at position 714–752 aa that was previously shown to be exposed on the surface of HAV particles (Robertson *et al.,* 1989). Surface exposure is generally considered to be an important indicator of potential antigenic reactivity (Emini *et al.,* 1985); however, among these peptides, only peptide 1307 was found to be immunoreactive (Table 1).

Immunoreactivity of peptides derived from nonstructural proteins

The antigenic composition of the entire HAV nonstructural area has not been previously reported. It is known, however, that the nonstructural proteins are antigenically reactive and may bind antibodies from anti-HAV-positive serum specimens (Jia *et al.,* 1992; Robertson *et al.,* 1992, 1993; Steward *et al.,* 1997). Nevertheless, no attempt has been made to map antigenic epitopes within these proteins.

P2A protein. Five peptides, 1315, 1316, 1317, 1318, and 1319, were synthesized to span the entire P2A protein (Fig. 1). Among these peptides, four were found to be immunoreactive (Table 2). All antigenically active P2A peptides could bind antibodies from 31.3 to 83.3% of acute-phase anti-HAV-positive serum specimens with average *P/N* values varying from 5.94 to 19.35 (Table 2). Peptide 1317 immunoreacted with 83.3% of acute-phase serum specimens and as such represents the most immunoreactive peptide found in the present study. Thus, the HAV nonstructural P2A protein is very antigenically reactive (Fig. 1). However, the P2A antigenic domain should be considered an extension to the C-terminal antigenic domain of the HAV VP1 protein since all overlapping consecutive peptides from 1312 to 1318 were strongly immunoreactive (Tables 1 and 2). Thus, the region spanned by these peptides at position 767–842 aa that comprise the C-terminal sequence of the VP1 protein and almost the entire P2A protein may be considered as one immunodominant domain of the HAV polyprotein (Fig. 1B, region 2).

P2B protein. Among 18 peptides derived from the HAV P2B protein, only 2 were found to be immunoreactive (Figs. 1D and 1E). These immunoreactive peptides identified two small independent antigenic domains at positions 922–941 and 961–980 aa (Table 2). Both peptides were very immunoreactive as evidenced by each peptide reacting with approximately 30.0% of acute-phase HAV serum specimens. The HAV P2B protein is the least immunoreactive region compared to all other HAV proteins, as assessed by the antigenic reactivity of synthetic peptides.

P2C protein. Among 30 peptides spanning this protein, 10 were found to be immunoreactive (Figs. 1D and 1E). These immunoreactive peptides identified six antigenic domains (Table 2). The N-terminal and the C-terminal domains at positions 1133–1152 and 1403–1422 aa, respectively, were identified with only one peptide each. Two internal domains at positions 1261–1280 and 1299–1318 aa were also identified with one peptide each. One domain at position 1210–1239 aa was identified with two peptides, while the domain at position 1331–1379 aa was spanned by four immunoreactive peptides (Table 2). None of the HAV P2C peptides was immunoreactive with more than 30.0% serum specimens. The most immunoreactive peptides, 1347, 1348, and 1367, were found to be immunoreactive with only 22.0–29.2% of sera (Table 2).

P3A protein. This protein contains only one antigenic domain (Table 2), located at the N-terminal region (Fig. 1B). Among six peptides spanning this protein, three were found to be immunoreactive; two of these peptides, 1368 and 1369, demonstrated strong immunoreactivity (Table 2). Consistent with the definition of an antigenic domain as presented in this paper, the P3A antigenic domain should be extended to include the C-terminus of the P2C protein, where immunoreactive peptide 1367 from the P2C protein is consecutive with peptide 1368 from the P3A protein (Table 2). Thus, as observed for the

TABLE 2

Antigenic Reactivity of Synthetic Peptides Derived from the HAV Nonstructural Proteins

Peptide	Sequence	Position aa	Acute[1] sera	P/N[2] acute sera	NHS[3]
P2A					
YK-1315	SHIECRKPYKELRLEVGKQR	792-811	26/48 54.2%	13.71 (3.1-75.61)	1/51
YK-1316	PYKELRLEVGKQRLKYAQEE	799-818	20/48 41.7%	8.71 (3.5-17.8)	2/51
YK-1317	QRLKYAQEELSNEVLPPPRK	810-829	55/66 83.3%	19.35 (3.47-125.8)	1/66
YK-1318	VLPPPRKMKGLFSQAKISLF	823-842	15/48 31.3%	5.94 (3.4-8.68)	0/51
P2B					
YK-1327	KVNFPHGMLDLEFIAANSKD	922-941	15/48 31.3%	6.16 (3.28-9.76)	1/51
YK-1331	KINLADRMLGLSGVQEIKEQ	961-980	13/48 27.1%	5.71 (3.71-10.3)	0/51
P2C					
YK-1341	NILKDNQQKIEKAIEEADEF	1133-1152	3/48 6.3%	5.23 (3.44-8.26)	1/51
YK-1347	LGSINQAMVTRCEPVVCYLY	1210-1229	13/48 27.1%	4.16 (3.18-6.2)	2/51
YK-1348	RCEPVVCYLYGKRGGGKSLT	1220-1239	14/48 29.2%	6.96 (3.66-15.48)	2/51
YK-1352	TKPVASDYWDGYSGQLVCII	1261-1280	8/48 16.7%	4.45 (3.38-5.81)	0/51
YK-1356	VSGCPMRLNMASLEEKGRHF	1299-1318	8/48 16.7%	5.49 (3.36-10.96)	0/51
YK-1360	NPSPKTVYVKEAIDRRLHFK	1331-1350	4/41 9.8%	4.08 (3.68-4.71)	0/46
YK-1361	VKEAIDRRLHFKVEVKPASF	1339-1358	5/41 12.2%	4.12 (3.21-6.28)	0/46
YK-1362	VKPASFFKNPHNDMLNVNLA	1353-1372	6/41 14.6%	7.82 (3.4-18.05)	0/41
YK-1363	KNPHNDMLNVNLAKTNDAIK	1360-1379	5/41 12.2%	5.96 (5.31-6.33)	0/41
YK-1367	VMTVEIRKQNMTEFMELWSQ	1403-1422	9/41 22.0%	7.82 (4.18-16.26)	1/41
P3A					
YK-1368	SQGISDDDNDSAVAEFFQSF	1421-1440	26/41 63.4%	9.58 (4.27-19.82)	1/27
YK-1369	DSAVAEFFQSFPSGEPSNSK	1430-1449	24/41 58.5%	21.71 (3.8-42.62)	1/27
YK-1370	FQSFPSGEPSNSKLSGFFQS	1437-1456	10/41 24.4%	8.05 (3.05-18.48)	1/27
P3B					
YK-1374	HGVTKPKQVIKLDADPVESQ	1500-1519	18/41 43.9%	10.57 (3.28-29.07)	0/27
P3C					
YK-1376	GLVRKNLVQFGVGEKNGCVR	1526-1545	3/41 7.3%	6.71 (3.8-12.77)	0/27
YK-1382	DVVLMKVPTIPKFRDITQHF	1603-1622	1/41 2.4%	8.4	0/27
YK-1388	MEEKATYVHKKNDGTTVDLT	1656-1675	4/41 9.8%	4.29 (3.99-4.7)	0/27
YK-1389	KNDGTTVDLTVDQAWRGKGE	1666-1685	3/41 7.3%	10.33 (5.53-18.26)	0/27
YK-1390	RGKGEGLPGMCGGALVSSNQ	1681-1700	10/41 24.4%	6.62 (3.52-13.98)	1/27
YK-1393	YAKLVTQEMFQNIDKKHESQ	1719-1738	24/41 58.5%	10.15 (4.64-16.36)	1/27
P3D					
YK-1394	RIMKVEFTQCSMNVVSKTLF	1739-1758	13/41 31.7%	6.98 (4.05-12.93)	3/27
YK-1395	FTQCSMNVVSKTLFRKSPIY	1745-1764	8/41 19.5%	6.52 (3.51-17.52)	1/27
YK-1399	MLSKYSLPIVEEPEDYKEAS	1791-1810	5/41 12.2%	6.86 (4.05-16.46)	1/27
YK-1407	LDENGLLLGVHPRLAQRILF	1866-1885	2/41 4.9%	3.43; 3.98	0/27
YK-1411	CPKDELRPLEKVLESKTRAI	1903-1922	8/41 19.5%	8.18 (4.65-17.65)	0/27
YK-1412	SKTRAIDACPLDYSILCRMY	1917-1936	10/41 24.4%	6.04 (3.4-15.15)	2/27
YK-1418	KTMIRFGDVGLDLDFSAFDA	1969-1988	6/41 14.6%	4.5 (3.35-6.21)	1/27
YK-1419	DLDFSAFDASLSPFMIREAG	1980-1999	5/41 12.2%	5.27 (3.98-8.49)	1/27
YK-1424	INNVNLYYVFSKIFGKSPVF	2052-2071	3/41 7.3%	6.69 (3.48-10.83)	0/27
YK-1429	LGMTATSADKNVPQLKPVSE	2112-2131	5/41 12.2%	10.25 (4.92-21.38)	0/27
YK-1434	SEKTIWSLIAWQRSNAEFEQ	2151-2170	11/41 26.8%	7.47 (3.85-16.03)	1/27
YK-1435	SLIAWQRSNAEFEQNLENAQ	2157-2176	6/41 14.6%	8.96 (5.16-13.71)	1/27
YK-1436	WQRSNAEFEQNLENAQWFAF	2161-2180	4/41 9.8%	4.49 (3.35-5.89)	1/27

Note. Alternating boldface and italic type represent individual antigenic domains.

[1] The number of positive serum samples divided by the total number of tested sera and percentage of immunoreactive sera.

[2] Mean *P/N* value; number in parentheses indicates the range of *P/N* values.

[3] Normal human sera.

P2A protein, the P3A protein contains one very strong antigenic domain that extends into the C-terminus of the preceding protein.

P3B protein. This short 23-aa protein was spanned by only one peptide, 1374 (Fig. 1), which demonstrated very broad and strong antigenic reactivity (Table 2). This peptide and those derived from P2A and P3A proteins represent the most diagnostically relevant peptides identified in this study.

P3C protein. Among 19 peptides synthesized spanning the entire P3C protein, 6 were immunoreactive (Figs. 1D and 1E). One peptide, 1393, derived from the very C-terminus was found to be one of the most immunoreactive peptides described in this study (Table 2) as evidenced by its reacting with almost 60.0% of acute-phase anti-HAV-positive serum specimens. The HAV P3C protein contains four antigenic domains, three of which were identified with one peptide each.

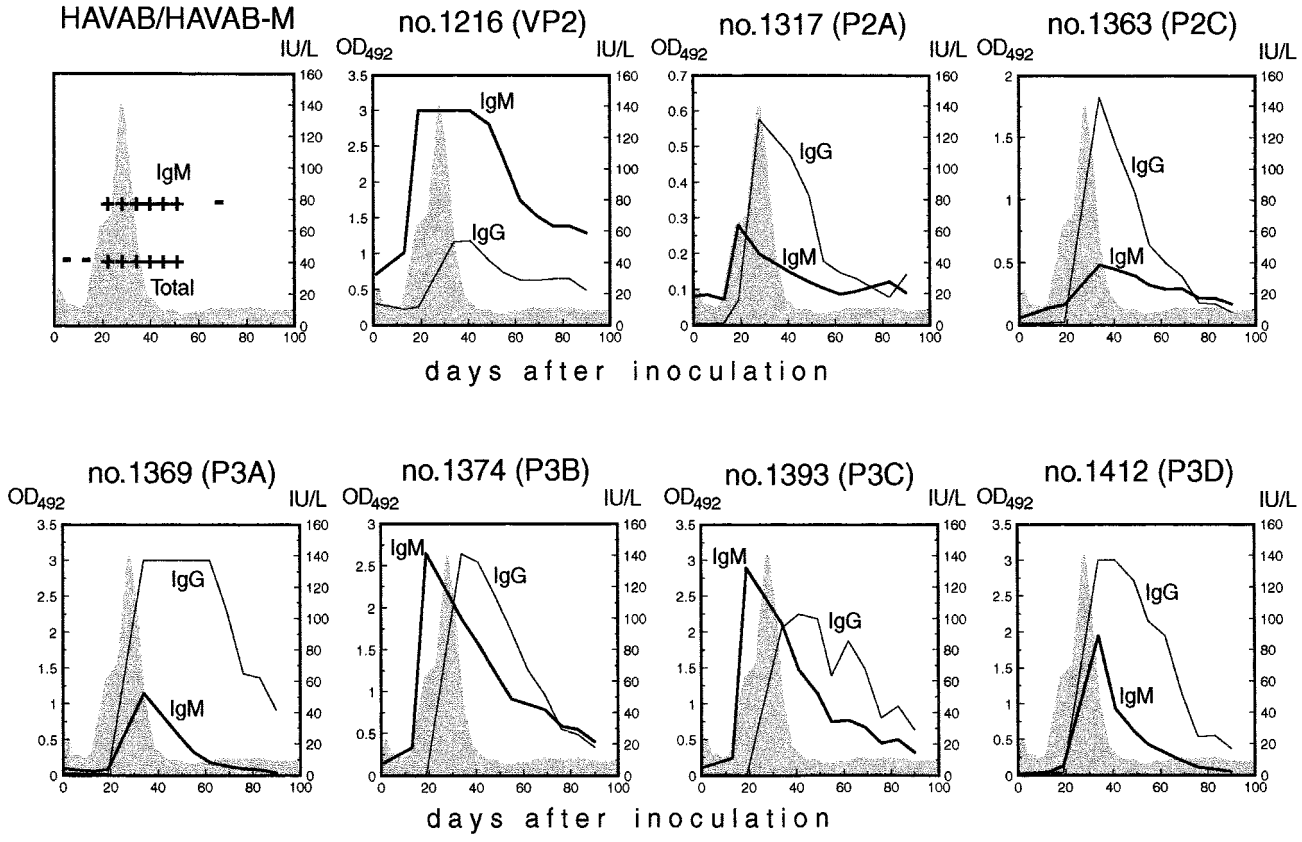


FIG. 2. IgG and IgM anti-HAV immunoreactivity of synthetic peptides with a seroconversion panel obtained from an experimentally HAV-infected chimpanzee 1357. Shaded area shows the ALT level. HAVAB/HAVAB-M panel shows the results for the detection of IgM and total antibodies with HAVAB-M and HAVAB diagnostic tests, respectively; plus and minus signs indicate positive and negative results of testing; the other specimens were not tested.

One domain was identified with three peptides (Table 2).

P3D protein. Forty-eight peptides were derived from this protein; 13 were found to be immunoreactive (Fig. 1). These immunoreactive peptides identified eight antigenic domains (Table 2). Four domains were identified with one peptide each, three were identified with 2 peptides each, and one was identified with 3 peptides (Table 2). Peptide 1394 was the most immunoreactive among all the P3D peptides. This peptide immunoreacted with approximately 32.0% of anti-HAV-positive acute serum specimens (Table 2). Similar to the P2A and P3A proteins, the P3D N-terminal antigenic domain may be extended into the C-terminal region of the P3C protein (Table 2).

Immunoreactivity of peptides with HAV seroconversion panels

All synthetic peptides presented in Tables 1 and 2 were tested with seven anti-HAV seroconversion panels obtained from HAV-infected patients ($n = 2$) and from experimentally infected chimpanzees ($n = 5$; see Materials and Methods). A specific seroconversion pattern of antibody binding was detected for all peptides. This finding demonstrates that the peptides shown in Table 1 and 2 specifically detected anti-HAV antibodies. The majority of the peptides demonstrated both IgG and IgM anti-HAV activity. A few examples of specific reactivity for representative peptides from each HAV protein, with follow-up serum specimens obtained from experimentally infected chimpanzee 1357 and with seroconversion panel RP-004 obtained from an HAV-infected patient, are shown in Figs. 2 and 3, respectively. It is interesting to note that IgM antibody can be detected with synthetic peptides for approximately the same period of time as with cell culture-derived antigen used in the commercially available test (HAVAB-M, Abbott Laboratories), whereas IgG anti-HAV activity can be detected for a much shorter period compared to HAVAB (Figs. 2 and 3). This observation may be explained by a relatively lower immunoreactivity with convalescent-phase anti-HAV-positive serum samples of even strongly immunoreactive peptides such as peptide 1317 (data not shown) in concordance with previous observations using synthetic peptides and convalescent-phase serum specimens (Bosch *et al.,* 1998; Chikin *et al.,* 1991; Kulik *et al.,* 1994, 1995).

Almost all synthetic peptides demonstrated a uni-

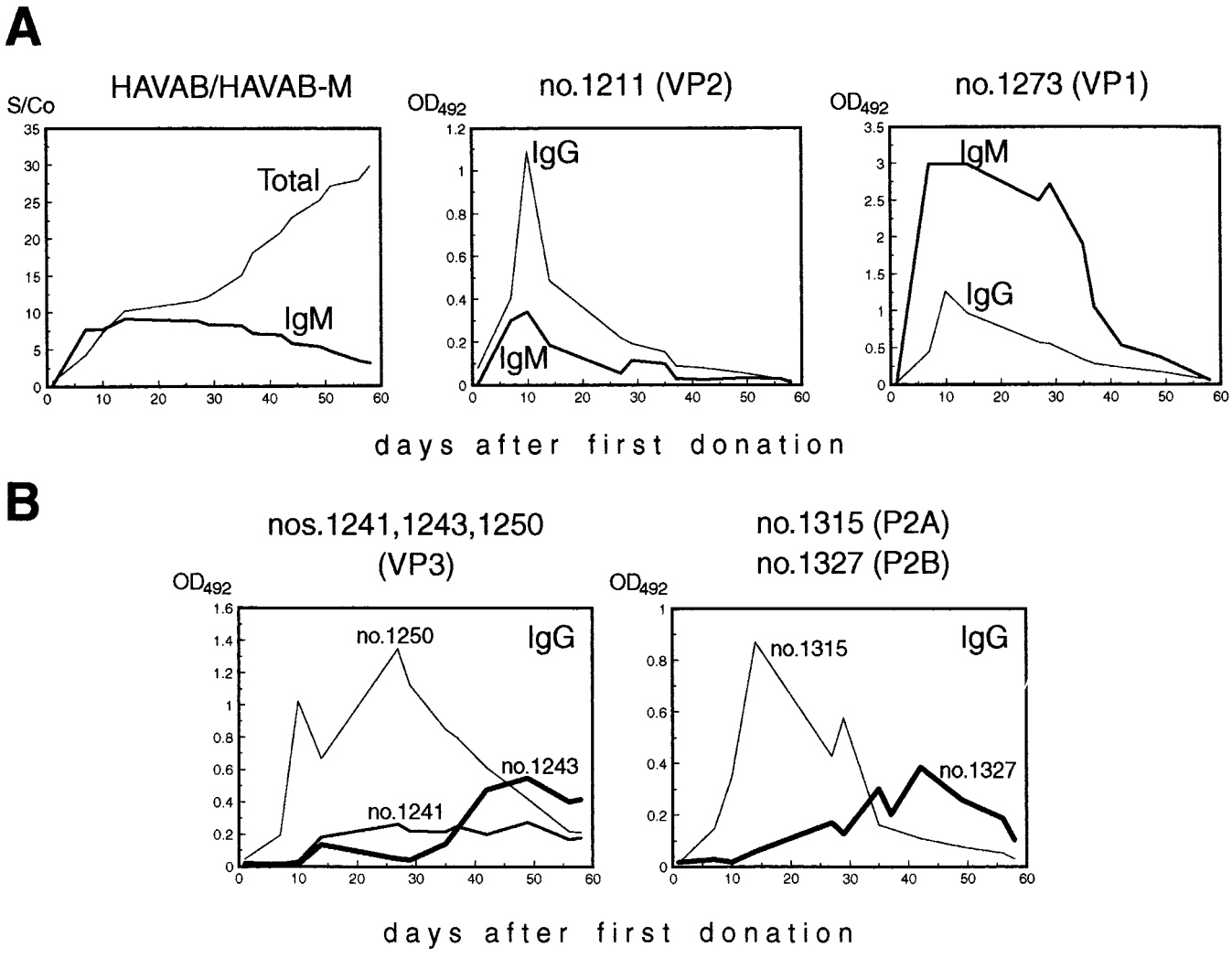


FIG. 3. IgG and IgM anti-HAV immunoreactivity of synthetic peptides with the seroconversion panel RP-004. HAVAB/HAVAB-M panel shows results for the detection of IgM and total antibodies with HAVAB-M and HAVAB diagnostic tests, respectively.

form pattern of immunoreactivity with the various seroconversion panels, as can be seen in Figs. 2 and 3A. Some peptides, however, showed a slightly different pattern of immunoreactivity (Fig. 3B). For example, peptides 1250 and 1315 (Fig. 3B) showed a typical reactivity with the seroconversion panel RP-004, which is very similar to the patterns seen with other peptides in Figs. 2 and 3A; by contrast, peptides 1243 and 1327 showed a "delayed" immunoreactivity pattern. It is interesting to note that peptides 1241, 1243, and 1250, which all belong to the VP3 protein, demonstrated two distinct patterns of immunoreactivity, typical and delayed (Fig. 3B).

Antigenic composition of the HAV polyprotein

In the present study a number of antigenic epitopes were identified by using synthetic peptides (Tables 1 and 2). These antigenic epitopes were modeled with 75 peptides almost uniformly scattered across the entire HAV polyprotein (Fig. 1). As mentioned above, some antigenic epitopes from the structural proteins had been previously discovered (Bosch *et al.,* 1998; Emini *et al.,* 1985; Kulik *et al.,* 1994, 1995), whereas none of the nonstructural antigenic epitopes had been previously identified. The finding of strong antigenic epitopes within the nonstructural proteins corroborates previous observations that antibodies specific to nonstructural proteins may be detected in anti-HAV-positive serum samples with protein products expressed in an *in vitro* transcription–translation system (Jia *et al.,* 1992; Robertson *et al.,* 1992, 1993; Stewart *et al.,* 1997).

By the use of synthetic peptides, 40 antigenic domains were found scattered along the entire polyprotein, and antigenic epitopes were identified within every HAV protein. An assessment of the antigenic reactivity of peptides reveals that, in general, structural proteins showed an average antigenic reactivity, whereas nonstructural proteins such as P2A, P3A, and P3B are very antigeni-

cally reactive (Figs. 1D, 1E, and 1F). Protein P2B, however, is the least immunoreactive (Fig. 1B). Approximately 30 of 75 immunoreactive peptides demonstrated a percentage immunoreactivity (Fig. 1D) and an average *P/N* value (Fig. 1E) greater than the average value calculated for all immunoreactive peptides. Only 13 peptides (1211 from VP2, 1266, 1312, 1313, and 1314 from VP1, 1315, 1316, and 1317 from P2A, 1368, 1369, and 1370 from P3A, 1374 from P3B, and 1393 from P3C) were found to exceed the average level for both parameters (Figs. 1D and 1E). Among these 13 peptides, only 9 (1211, 1313, 1314, 1315, 1317, 1368, 1369, 1374, and 1393) were identified with an antigenic index (see Materials and Methods) that was two times greater than the average value for the entire polyprotein (Fig. 1F). These 9 peptides belong to five antigenic domains (Fig. 1B). Domain 1, which is the only structural domain among the five strong antigenic domains, is located at position 57–90 aa. Domain 2, at position 767–842 aa, encompasses the C-terminal region of the VP1 protein and the entire P2A protein. Domain 3 contains the C-terminal region of the P2C protein and the N-terminal region of the P3A protein at position 1403–1456 aa. Domain 4 contains almost the entire P3B protein at position 1500–1519 aa. Domain 5 contains the C-terminal region of the P3C protein and the N-terminal region of the P3D protein at position 1719–1764 aa (Fig. 1B, Tables 1 and 2). It is interesting to note that three of the five strong antigenic domains such as domains 2, 3, and 5 contain the predicted protease cleavage sites (Fig. 1B). Another interesting observation is that domains 2, 3, and 4 belong to the smallest HAV proteins, P2A, P3A, and P3B. All five strong antigenic domains are hydrophilic (Fig. 1H) and are folded into alpha-helices separated by strong beta-turns (Fig. 1G), as predicted using computer-assisted analysis of secondary structure (Chou, 1990). Domain 2, the most immunoreactive domain, comprises the most hydrophilic region, which is also part of the largest predicted alpha-helical region within the HAV polyprotein (Figs. 1G and 1H).

An analysis of the immunoreactivity of synthetic peptides with HAV seroconversion panels demonstrated that both IgM and IgG antibodies can be detected with synthetic peptides for a short period of time around the acute phase of HAV infections (Figs. 2 and 3). A similar pattern of reactivity was found for peptides derived from both structural and nonstructural proteins. This finding suggests that several HAV-specific antigenic epitopes, which can be efficiently modeled with synthetic peptides, elicit antibodies for only a short time. Alternatively, synthetic peptides, because of some innate property such as low avidity of antibody binding, can efficiently detect anti-HAV antibodies only during the acute phase of infection. Recently, a similar short-term antibody response was observed with a recombinant P3C protein (Stewart *et al.,* 1997). This observation, taken together with our findings, strongly suggests that the immune response to the HAV nonstructural proteins is usually of short duration. Nevertheless, synthetic peptides or antigenic epitopes identified in the present study may have potential diagnostic application, especially for the detection of acute HAV infections currently diagnosed by the detection of IgM anti-HAV using cell-culture-derived virus as the antigenic target. Recombinant proteins or synthetic peptides from the structural proteins have not been successfully used as alternative sources of antigen in the development of enzyme immunoassays for the detection of anti-HAV because of apparently poor antigenic reactivity. Poor performance of these antigens has been explained by the strictly conformational nature of HAV antigenic epitopes (Lemon, 1992). In addition, current commercially available assays are unable to discriminate between natural infections and vaccine-induced immunity (Jia *et al.,* 1992; Robertson *et al.,* 1992, 1993; Stewart *et al.,* 1997). Antigenic domains discovered within the HAV nonstructural proteins may be most suitable for developing such an assay. Finally, data derived in the present study may be used to construct recombinant proteins or artificial antigens similar to the mosaic hepatitis E antigen (Khudyakov *et al.,* 1994) and used as antigenic targets in the development of diagnostic tests for the detection of acute HAV infections.

MATERIALS AND METHODS

Synthetic peptides

Peptides were synthesized by FMOC chemistry (Barany and Merrifield, 1980) on an ACT Model MPS 350 multiple peptide synthesizer (Advanced Chemtech, Louisville, KY) according to the manufacturer's protocols. After characterization by amino acid analysis, high-performance liquid chromatography, and capillary electrophoresis, peptides were characterized by enzyme immunoassay.

Serum samples

All anti-HAV-positive serum samples collected from anti-HAV-positive patients with acute liver disease and anti-HAV-negative serum samples collected from normal blood donors were randomly selected from a collection reposited at the Centers for Disease Control and Prevention (Atlanta, GA). All serum specimens were initially tested by commercially available kits for the presence of IgG and IgM anti-HAV activity (HAVAB and HAVAB-M, Abbott Laboratories, North Chicago, IL).

Enzyme immunoassay for anti-HAV

Synthetic peptides (110 μl) at a concentration of 10 μg/ml in 0.1 M phosphate-buffered saline (PBS), pH 7.5, were adsorbed to microtiter wells (Immulon II, Dynatech Industries, Inc., McLean, VA) at room temperature for 12 h. Serum specimens were diluted in PBS containing

0.1% Tween 20 and 10% normal goat serum (PBS-T); 100 μl of diluted specimens was added to each well, incubated for 60 min at 37°C, and washed with PBS containing Tween 20. Two different dilutions of specimen and detector antibodies were used. One set of serum specimens was diluted 1:100. The antibody binding was detected by adding 100 μl of affinity-purified anti-human IgG coupled to horseradish peroxidase (HRP) (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:30,000 and by incubating it for 1 h at 37°C. The second set of serum specimens was diluted 1:1000. In this case, 50 μl of affinity-purified anti-human IgG conjugated to biotin diluted 1:8000 and 50 μl of streptavidin coupled to HRP were added to microtiter wells and incubated for 30 min at 37°C. Two different cut-offs were established to identify anti-HAV specific immunoreactivity. The first cut-off, expressed as a *P/N* ratio and equal to 3.0, was statistically established as the mean of negative controls plus at least 3.5 standard deviations (SD) above the mean, where *P* represents the optical density value at 493 nm (OD_{493}) of anti-HAV-positive specimens and *N* represents the OD value of negative controls. An additional cut-off was used to ensure statistical reliability and accurate interpretation of positive results. In this case, in addition to anti-HAV-negative serum, two irrelevant synthetic peptides were used as supplemental negative controls to determine the degree of nonspecific binding of anti-HAV serum to synthetic peptides. One cut-off, expressed as a *P/N* ratio and equal to 3.0, corresponded to the mean of negative serum controls plus at least 3.5 SD above the mean. A second cut-off was also expressed as a *P/N* ratio, where *P* represents the OD value at 493 nm (OD_{493}) for HAV peptides immunoreacting with anti-HAV-positive specimens and *N* represents the OD value obtained with the irrelevant peptides. The second cut-off was equal to 2.0. Synthetic peptides were considered immunoreactive with anti-HAV serum specimens only when both criteria were satisfied. Tables 1 and 2 and Fig. 1 show *P/N* values obtained by using the anti-HAV-negative serum specimens.

The antigenic index, designated the A index (Fig. 1F), was derived by multiplying the mean of *P/N* values and percentage of serum specimens immunoreactive with each synthetic peptide (Tables 1 and 2) and therefore represents a measure of both the strength and the breadth of the immunoreactivity of each peptide with serum specimens. When antigenic properties of different peptides are compared, the A index can be calculated as the mean of *P/N* values for all anti-HAV-positive serum specimens tested with each peptide. Thus, the A index combines such parameters as the mean of *P/N* values of specimens tested positive with each peptide and the percentage of serum specimens immunoreactive with each peptide. In this study, because the mean of *P/N* values for specimens immunoreactive with each peptide and the percentage of immunoreactive sera were calculated long before, the A index was calculated as described above. This calculation gives different absolute value without affecting the relative value of the A index calculated as the mean of *P/N* in rating antigenic quality of synthetic peptides. We found that the A index in combination with such parameters as the average *P/N* of positive specimens and the percentage of immunoreactive serum specimens (Figs. 1D, 1E, and 1F) is a very helpful indicator of antigenic potential of peptides and as such was used in this study to measure the diagnostic relevance of each individual peptide.

HAV seroconversion panels

Two anti-HAV seroconversion panels, RP-004 and RP-013 (Profile Diagnostics, Inc., Sherman Oaks, CA), were used in this study. Panel RP-004 contains 15 members collected over 63 days from a patient with symptoms of hepatitis A infection including jaundice, malaise, dehydration, and elevated levels of alanine aminotransferase (ALT). Panel RP-013 also contains 15 members collected over 189 days from a patient with symptoms of hepatitis A infection.

Five seroconversion panels were obtained from experimentally HAV-infected chimpanzees (chimps 1357, 1402, 1439, 1487, and 1489). Chimp 1357 was injected intravenously with 10^6 chimpanzee infectious doses of wild-type HLD-2 HAV inoculum. Chimp 1402 was inoculated intravenously with 25 ng of live, attenuated virus (HAS-15, 160S fraction after sucrose gradient purification) without adjuvant, which resulted in no ALT elevation and no seroconversion. This animal was challenged with 1 ml wild-type virus (HLD-2) inoculated intravenously 218 days after the first inoculation. Chimps 1487 and 1489 were inoculated via an oral gastric tube with 1 ml SD-11 human stool suspension in veal broth (kindly provided by Dr. R. H. Purcell) containing 100 chimpanzee oral infectious doses. Chimp 1439 was inoculated intragastrically with a 10% stool suspension obtained from an experimentally HAV-infected cynomogus macaque (145), which resulted in no anti-HAV seroconversion and no ALT elevation, and was subsequently challenged with 1 ml wild-type virus (HLD-2) inoculated intravenously 145 days after the first inoculation. All animals exhibited ALT elevations and seroconverted following inoculation with HAV HLD-2. Twelve follow-up serum specimens collected from each animal over ~100 days after the inoculation were used to test the immunoreactivity of synthetic peptides in the present study.

Computer-assisted analysis

Amino acid sequence analysis was performed by using the Protean program from the Lasergene software package (DNASTAR Inc., Madison, WI). Hydropathic plots were built by the Kyte and Doolittle (1982) method

and protein secondary structure was predicted by the Chou and Fasman method (see Chou, 1990).

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